Neuroimmune consequences of postnatal ethanol exposure and the potential anti-inflammatory and pro-cognitive benefits of ibuprofen treatment

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Fetal alcohol spectrum disorders (FASD) afflict up to 5% of American school age children (May et al., 2009) and pose a significant public health problem. Alone, the most severe form of FASD, fetal alcohol syndrome, has been estimated to cost the USA more than $6.5 billion annually (Harwood, 2003). However, these estimates do not include a number of important cost drivers including education/vocational services for those with mild cognitive impairments and mental health care (Popova et al., 2011). Many of these individuals experience pervasive deficits in forebrain function including impaired attention and long-term memory (Jacobson et al., 2011; Rangmar et al., 2015), which may contribute to the increased incidence of mental illness, unemployment, and legal trouble seen in this population in later life (Streissguth et al., 2004).

In this ongoing study, rat pups are administered ethanol across postnatal days (PD) 4-9—a period of brain development comparable to the human third trimester (West et al., 1986). Our lab and others have demonstrated that FASD model rats are impaired in hippocampus-dependent tasks, including trace fear conditioning (TFC), in both adolescence and adulthood (Goodfellow et al., 2016; Schreiber and Hunt, 2013), which may be linked to impaired memory consolidation (Goodfellow and Lindquist, 2014). These rats also show diminished learning-induced phosphorylation of hippocampal ERK1/2 (DuPont et al., 2014), the activation of which is critical

[1] This work was conceptualized in conjunction with Dr. Derick Lindquist. Youn Ju Shin provided technical assistance.

for memory consolidation (Villarreal and Barea-Rodriguez, 2006), further indicating that ethanol exposure in early life can have long-term deleterious effects on the function of the hippocampus.

It has been suggested that ethanol-induced neuroinflammation is a factor in these physiologic and cognitive changes (Alfonso-Loeches and Guerri, 2011). Ethanol has been shown to act via toll-like receptor 4 (TLR4), a pathogen recognition protein that is critical for the activation of the innate immune system, through the induction of high-mobility group box 1 protein (HMGB1), an endogenous ligand for TLR4 (Sobesky et al., 2014). Downstream of TLR4, nuclear factor kappa B (NF_KB) and activator protein 1 (AP1) induce the activity of the cyclooxygenase (COX)-2 enzyme (Crews et al., 2006) which, in turn, increases the expression of pro-inflammatory cytokines (Alfonso-Loeches et al., 2010). This upregulation in cytokine production caused by the interaction of ethanol with TLR4 may negatively affect the developing brain (Fernandez-Lizarbe et al., 2009; Pascual et al., 2011). The neonatal rodent brain is especially sensitive to the effects of neuroinflammation due to decreased expression of antioxidants (Henderson et al., 1999) as well as the normal developmental processes that occur during this period that may be perturbed by altered neuroimmune signaling including: synaptic pruning (Liu et al., 2004), developmental cell death (Wakselman et al., 2008), and neurogenesis (Bland et al., 2010). Indeed, increased neuroinflammation following 3rd trimester-equivalent ethanol-exposure has been shown in mice on PD10 (Drew et al., 2015), demonstrating the acute effects of ethanol exposure during this period on neuroimmune activation.

In humans, maternal consumption of alcohol has also been linked to increased peripheral circulation of pro-inflammatory cytokines in both mother and fetus (Ahluwalia et al., 2000). Early-life exposure to increased cytokine expression can have a profound impact on cognition in later life (Bilbo and Schwarz, 2012; Rana et al., 2012), suggesting that neuroinflammation during

the ethanol exposure period may be an important contributor to cognitive dysfunction in 5E rats. Furthermore, neuroinflammation during early postnatal life may lead to lasting changes in the regulation and release of neuroimmune molecules such as IL-1β and TNF-α (Tiwari and Chopra, 2011; Williamson et al., 2011), which has the potential to interfere with the induction and/or maintenance of long-term potentiation and other forms of synaptic plasticity (Ross et al., 2003).

Ibuprofen is an over-the-counter, non-steroidal anti-inflammatory drug (NSAID) that is commonly used to treat pain, fever, and inflammation resulting from illness and/or injury. Its anti-inflammatory effects derive from its inhibition of the interaction between arachidonic acid and COX-2, the catalyzation of which is the rate-limiting step in pro-inflammatory signaling (Orlando et al., 2015). Research in rodents has demonstrated that ibuprofen, which easily crosses the blood-brain barrier (Parepally et al., 2006), is effective in attenuating increases in COX-2, IL-1β, and TNF-α and minimizing white matter damage in postnatal rat brains following ischemia (Carty et al., 2011). Furthermore, a study by Randall, Becker and Anton (Randall et al., 1991) demonstrated that ibuprofen administered alongside alcohol on gestational day 10 in mice significantly decreased the percentage of malformed fetuses and tempered fetal weight loss resulting from prenatal alcohol exposure, suggesting that ibuprofen may also mitigate the teratogenic effects of ethanol.

This experiment measures pro-inflammatory gene expression in the hippocampus of FASD model and control rats on PD10 and the efficacy of ibuprofen to reduce neuroinflammation during this critical period of brain development. A separate group of animals are subjected to behavioral testing as juveniles (i.e. PD31-33) to assess hippocampus-dependent learning and memory ability. If ibuprofen diminishes the expression of pro-inflammatory cytokines in early life, then we predict that learning and memory will be restored in juvenile FASD model rats.

Methods

Neonatal Ethanol Treatment

Long-Evans rats are derived from a breeding colony housed in The Ohio State University psychology vivarium. Litters are culled to a maximum of 12 pups on PD3 and a maximum of one male and one female from each litter are used per treatment group to minimize the impact of litter effects. Across PD4-9, a binge-like dose of ethanol is administered once daily (5 g/kg/day; **5E**) in a nutritive milk solution (22.66% vol/vol) via intragastric intubation (0.02778 mL/g). A second milk-alone intubation is given 2 h later to help maintain body weight in 5E pups who may nurse less due to inebriation. To control for the stress of the intubation, the control group (sham-intubated; **SI**) rats undergo the intragastric intubation procedure but do not receive any fluid. On PD4, blood samples from 5E and SI rats are collected via tail clip in heparinized capillary tubes (~20 µL) and centrifuged to isolate the plasma. An Analox GL5 Analyzer (Analox Instruments, Lunenberg, MA) is used to determine peak blood alcohol concentration (BAC) in the plasma from 5E rats; blood samples from SI rats are discarded.

Ibuprofen Treatment

Rats (SI & 5E) are subcutaneously injected with ibuprofen (IBU; 100 mg/kg on PD4 followed every 24 h by 50 mg/kg through PD9 (Carty et al., 2011)) or an equal volume of sterile phosphate buffered saline vehicle (VEH).

qPCR

On PD10, animals are deeply anesthetized with isoflurane and then sacrificed via rapid decapitation (a separate group of animals are used for behavioral analysis). The hippocampus is dissected out and sored at -80°C until further analysis. RNA is isolated from tissue using a Total RNA Kit for tissue (IBI Scientific, Peosta, IA) and quantified (Synergy HT Multi-Detection

Microplate Reader, BioTek, Winooski, VT). cDNA is synthesized with a First-strand cDNA Synthesis Kit (Origene, Rockville, MD). qPCR is run at various temperatures (efficiency 90-110%; Bio-Rad, model CFX96, C1000 thermal cycler) optimized for each individual primer using Bullseye EvaGreen qPCR MasterMix intercalating dye (MidSci, St. Louis, MO). Results are quantified via the comparative cycle threshold ($2\text{-}\Delta\Delta C_t$) method, normalized to GAPDH. The following primer sequences are used (5'-3'; Integrated DNA Technologies, Coralville, IA): *Il1b* (Primer 1: GTGCTGTCTGACCCATGT, Primer 2: TTGTCGTTGCTTGTCTCTCC), *Tnf* (Primer 1: GACCCTCACACTCAGATCATCTTCT, Primer 2: TGCTACGACGTGGGCTACG), *Casp3* (Primer 1: CGTACAGTTTCAGCATGGC, Primer 2: CCGACTTCCTGTATGCTTACTC), and *Gapdh* (Primer 1: AACCCATCACCATCTTCCAG, Primer 2: CCAGTAGACTCCACGACATAC).

Behavior

Humans with FASD commonly demonstrate impairments in attention as well as both working and long-term memory (Fryer et al., 2007; Lee et al., 2004; Mattson et al., 2006; Mattson et al., 2011; O'Hare et al., 2009). As a result, trace fear conditioning (TFC), a challenging, forebrain dependent task that requires focused attention and memory (Beylin et al., 2001; Han et al., 2003; Knight et al., 2006), is an ideal test of cognitive function in FASD model rats. In TFC, rats must acquire (i.e. encode and consolidate) an association between a neutral conditioned stimulus (CS; tone) with a mildly aversive unconditioned stimulus (US; footshock) when a stimulus-free trace interval is interposed between the CS and US. The trace interval length determines the necessity of the hippocampus—trace intervals exceeding 5-10 s are hippocampus-dependent whereas trace interval shorter than 5 s are hippocampus-independent (Chowdhury et al., 2005; Misane et al., 2005). PD4-9 ethanol exposure has been shown to impair hippocampus-dependent TFC in both

adolescence and adulthood (DuPont et al., 2014; Goodfellow et al., 2016; Hunt et al., 2009; Schreiber and Hunt, 2013). As our particular interest is the assessment of hippocampal function, a 15 s trace interval will be employed in this experiment (see Figure 1).

On PD31, rats receive 10 presentations of a 15 s tone (2.8 kHz, 80 dB) followed 15 s later by a 1 s footshock (0.8 mA) with a 180 ± 30 s intertrial interval. On PD32, rats are returned to the conditioning context and, after a 120 s baseline period, observed for context-specific freezing behavior for 10 min. On PD33, rats are placed into a novel context and given 10 presentations of the 15 s tone (120 ± 30 s intertrial interval) without any footshocks; CS-evoked freezing behavior are assessed during each tone as well as during the 15 s following tone offset, mimicking the trace interval. During training and testing, freezing behavior is measured via an automated video analysis system (FreezeScan, CleverSys).

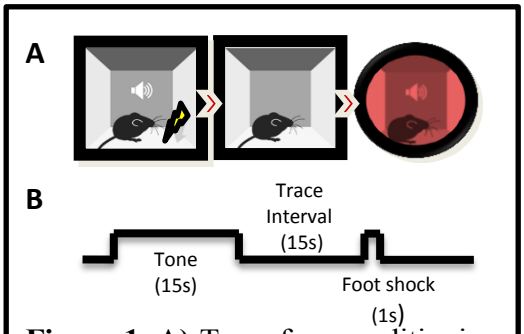

Figure 1. A) Trace fear conditioning and test sessions**, B)** a single conditioning session trial

Statistical Analysis.

Data are analyzed via single and multi-factorial analysis of variance (ANOVA) for neonatal treatment, drug effects, and learning performance. Significant ANOVAs are followed by appropriate post-hoc tests, including Bonferroni-corrected one-way ANOVAs and Tukey's post-hoc analyses. Outliers that are more than two standard deviations from the mean are excluded from further analysis (Dokovna et al., 2013; Weber et al., 2013).

Preliminary Results[2]

Blood Alcohol Concentration

Mean (±SE) peak BACs were 355±4.6. One-way ANOVA failed to reveal any significant effect of Drug ($p=0.33$), indicating that 5E rats achieved similar peak BACs, regardless of treatment with IBU or VEH.

Body Weight

PD4-10 body weights were analyzed via 2 (Treatment) x 2 (Drug) x 7 (Day) repeated measures ANOVA. Significant effects of Day ($F(6, 336)=1091.2$, $p<0.0001$), Drug x Day ($F(6, 336)=5.1$, $p<0.0001$), and Treatment x Day ($F(6, 336)=8.7$, $p<0.0001$), but not for Treatment x Drug x Day ($p=0.98$) were detected. Next, each day was analyzed separately via Bonferroni-corrected 2 (Treatment) x 2 (Drug) ANOVAs requiring, at 7 contrasts, $p<0.007$ for significance (maintaining family-wise $\alpha=0.05$). No significant main effects or interactions were noted, indicating that weight did not differ between treatment groups across PD4-10. To conserve animals, SI+IBU rats were not used for behavioral analysis as SI+VEH and SI+IBU rats had similar body weight and gene expression (see below).

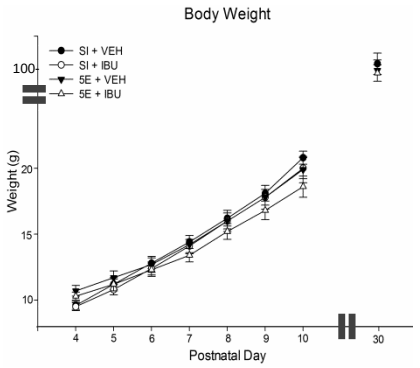

Figure 2. Body weight (mean±SE) across PD4-10 and on PD30 by ethanol and IBU treatment.

Weights were also measured on PD30 in rats submitted to TFC. A Treatment x Drug ANOVA failed to reach significance ($p=0.55$), indicating that behavioral results cannot be attributed to differences in body size.

[2] Note that data collection is still in progress and final results may significantly differ from those presented in this document.

Polymerase Chain Reaction

Il1b: A 2 (Treatment) x 2 (Drug) ANOVA revealed a significant effect of Treatment ($F(1, 31)=9.13$, $p<0.01$), Drug ($F(1, 31)=4.37$, $p<0.05$), and Treatment x Drug ($F(1, 31)=4.36$, $p<0.05$). Post-hoc analyses of the Treatment x Drug interaction found that 5E+VEH rats expressed significantly more *Il1b* than all other groups ($p<0.05$)—see Figure 3A. No differences were noted between SI+VEH, SI+IBU, and 5E+IBU.

Tnf: A 2 (Treatment) x 2 (Drug) ANOVA revealed a marginally significant effect of Treatment ($p=0.088$), Drug ($p=0.069$), and Treatment x Drug ($p=0.094$)—see Figure 3B.

Casp3: A 2 (Treatment) x 2 (Drug) ANOVA failed to reveal any significant effect of Treatment ($p=0.89$), Drug ($p=0.90$), or Treatment x Drug ($p=0.27$)—see Figure 3C.

Trace Fear Conditioning

Conditioning Session: One-way (Treatment) ANOVAs showed no differences in mean freezing behavior during the tone ($p=0.42$) or trace interval ($p=0.48$). Furthermore, no differences were noted for Drug during the tone ($p=0.81$) or trace interval ($p=0.75$). As expected, a repeated-measures ANOVA revealed a significant effect of Trial during the tone ($F(9, 225)=18.7$, $p<0.0001$) and trace interval ($F(9, 225)=33.6$, $p<0.0001$), indicating a change in freezing behavior across the conditioning session. No differences in Treatment x Trial or Drug x Trial interactions were noted.

Context Test: One-way ANOVAs showed no significant effect of Treatment ($p=0.69$) or Drug ($p=0.99$) in mean freezing behavior. The Treatment x Drug interaction failed to reach significance ($p=0.89$)—see Figure 3D.

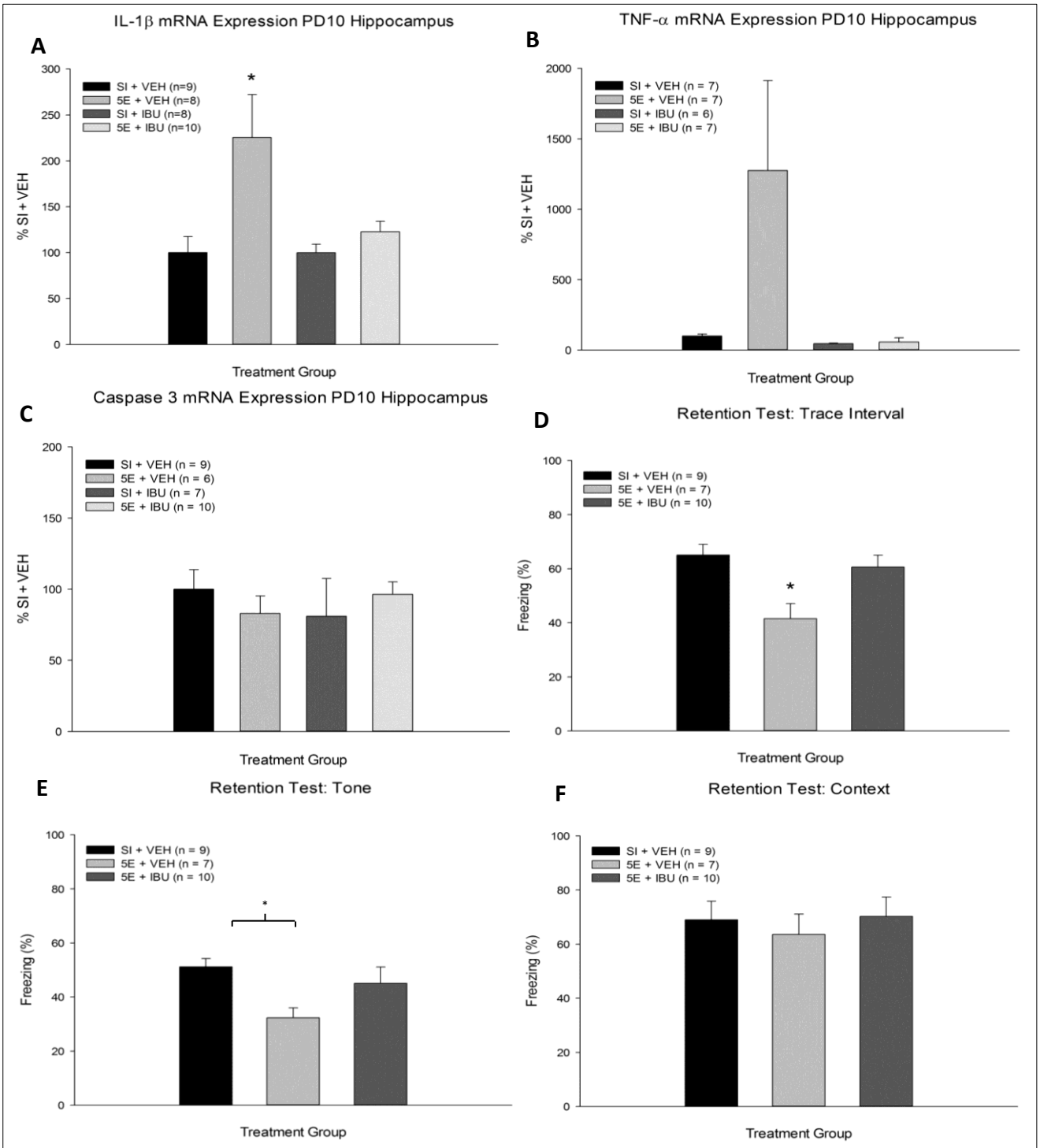

Figure 3. Hippocampal gene expression analysis via qPCR (mean±SE) relative to SI + VEH rats on PD10, collapsed across sex. **A)** IL-1β mRNA was significantly increased in 5E + VEH rats relative to all other groups. No significant effects were noted for **B)** TNF-α or **C)** caspase 3.
Mean percent freezing at test (±SE), collapsed across sex. **D)** A significant Treatment x Drug interaction was noted in freezing behavior to the trace interval with 5E + VEH rats freezing less than the other groups. **E)** A significant Treatment x Drug interaction was also found in freezing to the tone with 5E + VEH rats freezing less than SI + VEH rats. **F)** No differences were noted in freezing to the context. Significance ($p < 0.05$) denoted by an asterisk (*).

Tone Test (Tone): Freezing behavior was measured during the 60 s prior to the onset of the first trial; low levels of freezing for all groups indicate a successful context switch. Furthermore, ANOVAs failed to detect any differences in Treatment ($p=0.98$), Drug ($p=0.07$), or Treatment x Drug ($p=0.10$). During the tone presentations, no significant Drug effects ($p=0.76$) were found but a significant Treatment effect was noted ($F(1, 25)=4.6$, $p<0.05$), with 5E rats impaired relative to SI rats. A significant Treatment x Drug interaction was also revealed ($F(2, 24)=4.3$, $p<0.05$). Post-hoc analysis showed that 5E+VEH rats showed significantly less freezing behavior than SI+VEH rats but did not differ from 5E+IBU rats. 5E+IBU rats also did not differ from SI+VEH rats—see Figure 3E.

Tone Test (Trace Interval): One-way ANOVAs of freezing during the trace interval presentations showed no effects of Treatment ($p=0.07$) or Drug ($p=0.32$) but a significant Treatment x Drug interaction was detected ($F(2, 24)=6.2$, $p<0.01$). Post-hoc analysis showed that 5E+VEH rats froze significantly less than SI+VEH and 5E+IBU rats. 5E+IBU rats did not differ from SI+VEH rats—see Figure 3F.

Expected Outcomes

Preliminary data from our lab (Figure 3A-C), demonstrates that ethanol treatment increases pro-inflammatory gene expression on PD10 (IL-1β and TNF-α but not caspase 3) but that ibuprofen co-treatment may diminish this increased expression in 5E rats. Consistent with previous studies, 5E rats demonstrate reduced freezing following TFC at test (Schreiber and Hunt, 2013), particularly during the trace interval (Goodfellow et al., 2016). It is currently unknown if the effects of ibuprofen on inflammatory signaling may also rescue cognitive function though current data (Figure 3D-F) indicates that TFC freezing behavior at test may be restored in juvenile 5E rats following ibuprofen treatment.

Despite promising preliminary results, amelioration of neuroinflammation on PD10 may not result in improved TFC performance. In addition to neuroinflammation, prolonged ethanol exposure has also been demonstrated to induce excitotoxic cell damage following ethanol withdrawal (Hoffman and Tabakoff, 1994; Lovinger, 1993)—controlling neuroinflammation may not be sufficient to rescue cognitive function. Stress (e.g. the intubation procedure and injections) may alter pro-inflammatory signaling (Avitsur et al., 2013; Pugh et al., 1999), making the SI group critical for comparison to 5E rats when assessing both biochemical and behavioral effects of ethanol and ibuprofen. If this is the case, ibuprofen is predicted to reduce neuroinflammation and improve performance on TFC in SI rats as well.

While the potential benefits of ibuprofen to the mother and/or child may outweigh its risks, it is currently classified as a pregnancy category C-D drug. In rats, ibuprofen can induce skeletal abnormalities, hepatotoxicity and changes in peripheral hormonal signaling (Burdan, 2004; Castell et al., 1988; Gebrekristos et al., 2010). Controlled studies in healthy human newborns are lacking though, due to its efficacy in treating the heart condition patent ductus arteriosus (PDA) following premature birth (Ohlsson et al., 2015), some data does exist on the effects of ibuprofen on pre-term infants. Adverse effects may include impaired renal function, gastrointestinal disorders, and respiratory distress (Keady and Grosso, 2005). That said, studies in children under the age of 2 years indicate that serious side-effects associated with ibuprofen are rare (Lesko and Mitchell, 1999). With this in mind, positive results with ibuprofen would indicate the potential of anti-inflammatory therapy for the restoration of cognitive function in FASD and may promote the investigation of other agents (e.g. infliximab) that are safer for use during pregnancy and after birth (Khan et al., 2014).

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